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Biofilm management in paper and packaging board manufacturing process

Bacterial community analysis by DNA technology

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Tiivistelmä

Paperi ja kartongin valmistus prosessit ovat avoimia ja ravinnerikkaita. Nämä olosuhteet suosivat biofilmien kasvua prosesseissa. Energian säästötavoitteet ja kemikaalien käytön vähentäminen tuotantoprosesseissa kasvattavat biofilmien muodostumisen riskiä. Biofilmien lisääntyminen johtaa tuotantohäviöihin, laitteiston ja putkiston kulumiseen sekä työturvallisuuden heikkenemiseen. Biofilmit aiheuttavat myös lopputuotteen likaantumista joka johtaa asiakasvalituksiin ja reklamaatioihin. Tutkimus toteutettiin osana Stora Enson strategista BioM-projektia ja tulokset liitettiin osaksi Stora Enson ”Microbial Management Program” -strategiaa

Tutkimuksen tarkoituksena oli selvittää biofilmien syntymekanismeja ja tutkia mahdollisuuksia vähentää biofilmin muodostumista tuotantoympäristössä. Tutkimusta varten valitulta kartonkikoneelta eristettiin roiskealue jonka biofilmikehittymistä seurattiin uusilla DNA-pohjaisilla menetelmillä. Tutkimus suoritettiin mittaamalla biofilmin määrää kvantitatiivisesti (qPCR) ja syntyneen biofilmin bakteeriyhteisöä kvalitatiivisesti (NGS).

Tutkimuksessa havaittiin että biofilmin muodostuminen (primääritartunta) alkaa tunneissa. Ensimmäisinä tarttujina olivat a-proteobakteerit. Biofilmin bakteeriyhteisö monimuotoistuu ensimmäisten viikkojen ja kuukausien aikana ja kypsyy (maturoituu) noin vuoden kuluessa.

Biofilmien muodostuminen on monimutkainen dynaaminen prosessi jonka hallintaan tarvitaan useita toimintamalleja. Näitä ovat prosessiolosuhteiden oikea hallinta, mekaaninen kunnossapito sekä kemiallinen torjunta. Tärkeimpänä loppupäätelmänä oli, että vain oikeilla mittaustavoilla voidaan ohjata näitä toimintamalleja tehokkaampaan suuntaan.

Avainsanat (asiasanat) Biofilmi, Next generation sequencing, qPCR, pakkauskartonki

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Abstract

Board and paper manufacturing processes are open and nutrient rich. These circumstances promote the growth of biofilm in the processes. Reductions in energy and chemical (biocide) consumption increases the risk of biofilm formation. Formation of biofilm leads to decrease in production efficiency, weathering and corrosion of machinery and piping and increase in occupational health risks. Biofilm formation reflects to the end product quality due to dirt spots, taint and odour issues which are identified by customer reclamations.

The research of biofilm management in paper and packaging board manufacturing processes was executed as a part of Stora Enso's strategic BioM-project. The results from the research were added as a part of Stora Enso "Microbial Management Program".

Purpose of the research was to identify the biofilm formation and investigate the possibilities to reduce the formation in production environment. One Stora Enso packaging board machine was selected for the research. Area of the wet end spatter area, where biofilm formation has been identified, was restricted for the study. The area was measured for biofilm formation with DNA based methods i.e. next generation sequencing and quantitative polymerase chain reaction.

Results indicate that the primary formation of biofilms is initiated by the attachment of a-proteobacteria. The primary attachment occurs within hours of cleaning the area and develops to mature biofilm within months up to a year.

Biofilm formation is a complex and dynamic process. Biofilm management requires multidisciplinary approach where process parameters adjustment, mechanical cleaning and the right use of biocides are combined in a right manner with guidance of relevant measurement techniques.

Keywords/tags (subjects) Biofilm, qPCR, NGS, DNA

Miscellaneous Confidential information must be marked clearly stating which appendixes are confidential and what the confidentiality is based on and how long the period of secrecy is. For example: ***attachments 1, 2, 3, 4, 5 and 6 are confidential which have been removed from the public thesis. Grounds for secrecy: Act on the Openness of Government Activities 621/1999, Section 24, 17: business or professional secret. Period of secrecy is five years and it ends 18.5.2025.***

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Abbreviations

16s rRNA – 16 subunit ribosomal ribonucleic acid

DNA – Deoxyribonucleic acid

HTS – High Throughput Sequencing

MMM – Molecular Microbiological Methods

NGS – Next Generation Sequencing

PCR – Polymerase Chain Reaction

qPCR – quantitative Polymerase Chain Reaction

RDB – Ribosomal Database

SE MMP - Stora Enso Microbial Management Program

1 Introduction

1 Biofilms and their management in industrial processes

Paper and board mills are open systems. These open systems provide favorable conditions for microbial growth. Microbial growth in the system may cause economical losses (Torres et al. 2012, 199-206). These losses include deterioration of raw materials, reduced product quality (See Figure 1.), interference with production processes such as web-brakes (See Figure 4.) and the increase risk of occupational hazard occurrence (Principea et al. 2017, 485-495). In addition, increase in the system chemicalization and production of waste material increases demands in wastewater treatment systems. These economical, occupational hazard and impurity losses are caused by the formation of odorous products, discoloration, and the formation gaseous compounds by microbial activity in the production processes (Flemming et al. 2013). More importantly, the microbial growth may contaminate the end product *i.e* liquid packaging board, which in return poses a risk for the consumers of the packed goods in form of toxin producing bacteria such as *Bacillus cereus* (Pirttijärvi et al. 2000, 231-239).

Chlorine-free bleaching, processing at neutral pH, closed water cycles, and the use of recycled paper are emerging trends in paper and board machine operations. These trends might favor microbial growth and biofilm formation in the process environment. Paper and board manufacturing today is characterized by increased closure of the water system, conversion to neutral/alkaline systems, changes in chemical additives, and increased recycled fiber use, all of which have effects on the microbiological effects and decrease mill performance. Running the process under closed conditions leads to accumulation of nutrients and higher oxygen demand. These changes poses the risk of anaerobic and reduced redox in the system. This favors fermenting organisms that form low-chain fatty acids, causing malodors. Under such conditions, microbially influenced corrosion is also favored and this can affect electrical control and weathering of metal surfaces which again influences the operation conditions. (Bajpai 2015, 43-61)

Problem associated with biofilms is the extensive extracellular polymeric substances, which is composed of a large variety of highly hydrated polysaccharides, proteins, nucleic acids, and lipids. Furthermore biofilm provides favorable environment for pathogenic bacteria to thrive. Biofilm itself acts as a protective environment against biocides, thus, exposing cumulative risk in the production environment (Declerck et al. 2009, 593-603; Kolari et al. 2003, 225-238). No 'silver bullet' against biofouling can be expected, and effective countermeasures have to be based on holistic approaches (Flemming et al. 2013).

Figure 1. End product impurities. Example of impurities in end product and the bacterial DNA profiles obtained by qPCR. When the process has been profiled the impurites can be linked to the contamination source (Attachment 5).

Bajpai (2015, 54) explains that "the development of biofilm at a surface is the net result of several physical, chemical, and microbial processes… Several factors affect biofilm development including temperature, pH, oxygen levels, hydrodynamics, osmolarity, and the presence of specific ions, nutrients, and factors derived from the biotic environment. The combination of these effects eventually determines the pattern of behavior of a given bacterium with respect to biofilm development."

Purpose of the research was to identify the biofilm formation and investigate the possibilities to reduce the formation in production environment. One Stora Enso packaging board machine was selected for the research. Area of the wet end spatter area, where biofilm formation has been identified, was restricted for the study. Two DNA-based methods (quantitative polymerase chain reaction and next generation sequencing) were selected for the quantification and identification of bacteria

responsible for the primary attachment. Furthermore, as the biofilm formation is a complex and dynamic process biofilm management approaches were discussed.

2 Forms of biofilm

Biofilms differ in relation to degree of maturation, thickness, bacterial density and diversity, as well as physicochemical characteristics, diffusion characteristics and rheological properties. Board and papermaking deposits can be classified as being organic, inorganic, or microbiological. Most paper machine deposits contain a combination of some or all of these major component types. Although inorganic deposits tend to be more of a problem in the stock and flow-piping systems of the paper mill, organic deposits tend to be more problematic in the headbox, wet end, and press section of the paper machine. These contaminants form tacky deposits that can plug forming fabrics and wet press felts and wires (Holik 2013, 983-1002). Microorganisms in paper mill systems are recognized as two forms: planktonic and sessile. Planktonic organisms do not readily cause problems in the system; however, in the sessile form, these same organisms can become problematic. Inorganic and organic deposits offer a readily available food source for sessile organisms, which attach themselves to deposit surfaces. Several of these organisms classified as slime formers are able to secrete a protective polysaccharide coating around themselves known as biofilm. The slime formers, in combination with inorganic and organic process additives and contaminants, can form deposits that coat process equipment and create sheet quality and runnability problems. In addition to slime formers, anaerobic bacteria can form under deposits. The most common type of anaerobic bacteria found in paper mills is sulfate-reducing bacteria, which is capable of converting sulfate in the system to highly corrosive hydrogen sulfide. (Holik 2013, 983-1002.)

The type of microorganisms encountered in the papermaking process is diverse. It includes aerobic spore-forming bacteria, aerobic non-sporulating, anaerobic bacteria, molds, yeasts and occasionally also algae (Kolari et al. 2003, 225-238). Several groups have studied the biofilms and found that most common of the biofilm producing bacteria is Aerobacter aerogenes which is found in paper mills and gives rise to a soft and gelatinous slime. Several variants of this species have been found, which are

mobile and can select the most appropriate location of the paper machine for forming colonies. Other bacterial species detected in slime have been Chlamydobacteriales, Alcaligenes, Arthrobacter, Proteus, Bacillus, Escherichia coli, Pseudomonads, and others. (Tiirola et al. 2009, 929-937) Bacteria most often found in paper and board machine slime include species of Flavobacterium, Clavibacter, Sphaerotilus, and Leptothrix (Lahtinen et al. 2006, 734-740). Peltola et al. (2013) have also investigated the primary formation of biofilms in board manufacturing processes and have found that genus Deinococci is the most prelevant microorganism in biofilm formation (Peltola et al. 2008, 1651-1657). These studies, eventhough contradicting, support the thesis of Bajpai (2015) that the population is diverse and generalization of the biofilmformation can not be done; each mill at given time expresses individual charasteristics of biofilm formation and, indeed diverse population dynamics. The nature of microorganisms developing in slime plays an important part in the location of slime on the machine. Most of mill infections are observed at a very localized, principal point where the bacterial proliferation is able to develop and from where the greater part of the other infections expand. The white water circuit has been identified as the center of infection in most of the cases (Bajpai 2015, 43-61).

3 Methods to investigate and control biofilm formation

The investigation of biofilm formation has not been easy and several different techniques have been developed; slime collection boards, identification of contaminated points, standard plate count method, rapid counts, adenosine triphosphate assay, Bio-Lert method, slime monitor, special sensors, optical fouling monitor and a specialized photometer unit for online biofilm monitoring. All of the mentioned methods have been assessed as the best available technique method for several studies. Recently the focus has been on DNA based methods and especially development of so called NGS, Next Generation Sequencing, methods. (Douterelo 2016, 3301-3311 ; Flemming 2002, 629-640; Henne 2012, 3530-3538.)

Successful strategies against biofouling should be based on integrated approaches that consider the entire system to be protected. Future studies should focus on the mechanism of deposit formation in paper machines in combination with the

development of deposit control agents to meet the industrial demands of efficiency as well as environmental sustainability. This might also include new treatment methods of stainless steel to prevent or influence the attachment mechanism of the microbes and other substances. Understanding and implementing of such studies could lead to less toxic antifouling products as well as individually designed, more cost-efficient programs for each paper machine in the future. (Flemming et al. 1996, 517-524)

Biofilms in the paper and packaging board process are reflected to the end product hygiene. In a way, the ultimate goal of paper and packaging board product hygiene management is to minimize the microbial contamination in end products. The relationship is, however, anticipated to be highly complex due to the dynamic nature of the microbial systems involved. Nevertheless, it is anticipated that stressing of bio-film will result in a defensive reaction within the biofilm so that microorganisms transform into resistant forms (heat resistant forms, spores, viable-but-non-cultivable bacteria). Novel approach is to prevent the formation of spores and other resistant forms of bacteria in biofilms so that no resistant bacteria from biofilms will contaminate the end product. (Zhou et al. 2015, 21711-21733)

State-of-the-art knowledge views biofilms as highly controlled and diversified ecosystems with similarities to multicellular organisms (Figure 2.). Biofilms have a polysaccharide structure (e.g. bacterial cellulose hydrogel membrane) as backbone which stabilizes microbes into air-water interface and act as a protective shield against changes in the environment. Furthermore, due to the nanoporous structure of the backbone, nutrients and metabolites freely diffuse around the growing population.

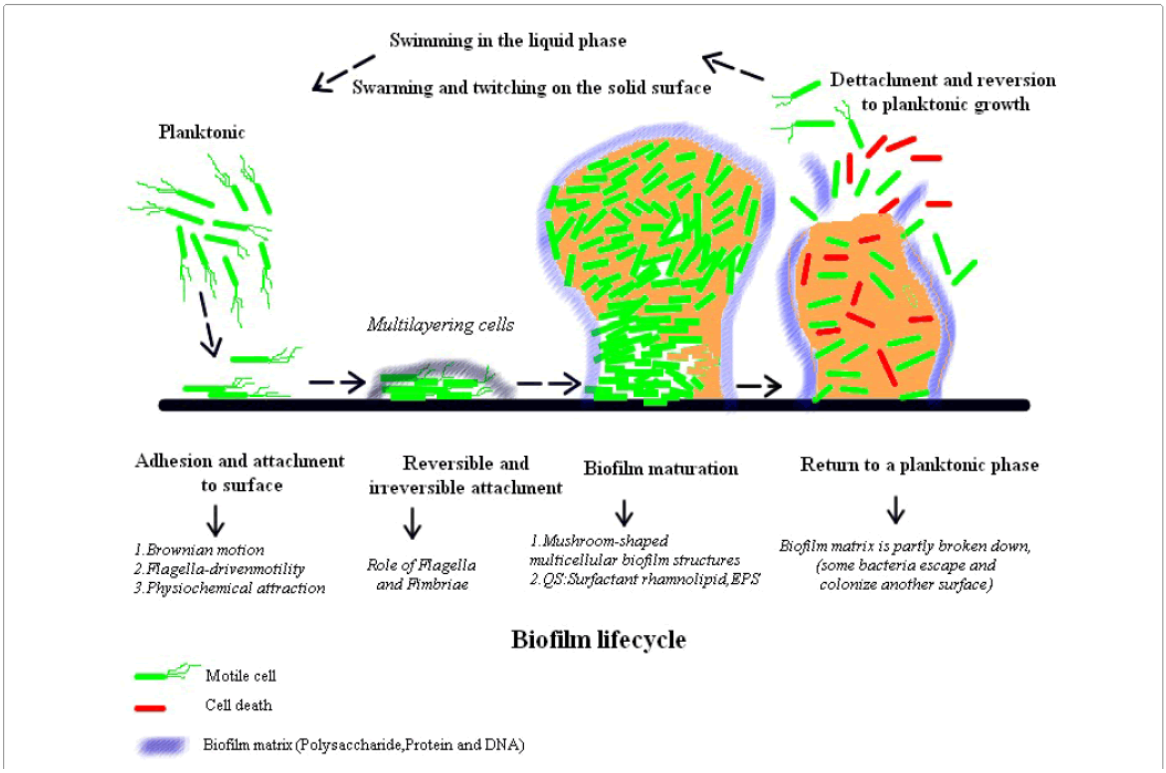

Figure 2. Biofilm formation**.** Schematic representation of biofilm dynamics starting from biofilm for-mation to its maturation and further to its death and detachment. (Meliani & Bensoltane 2015, 126)

Biofilm formation, maturation and destruction is regulated via quorum sensing signal molecules produced by bacteria. Only part of the quorum sensing signals are characterized but already now it is known that quorum sensing molecules include a wide range of compounds from homoserine lactone derivatives to complex peptides. Signal responses are still poorly understood and therefore, this project takes an experimental approach for elimination of biofilms. Prevention of biofilm formation and growth can be achieved by interfering with signals regulating onset of biofilms while interference with signals regulating biofilm maturation may result in increased sensitivity to biocides and washing procedures. Break-down of biofilms can be induced using interferences with signals regulating biofilm destruction. (Rutteford & Bassler 2012,)

While other industries *i.e.* the oil and gas industry, has witnessed increased applications of molecular microbiological methods (MMMs) for diagnosing and managing biofilm formation the process for establishing clear links between microbiological conditions and fouling mechanisms is still emerging. Different MMMs

provide various types of information about microbial diversity, abundance, activity and function, all of which are quite different from the culture-based results that are familiar to other industry professionals. In addition, a multidisciplinary process for establishing the significance of molecular microbiological data in regard to biofouling threat identification, mitigation and monitoring has yet to be clearly established. As a result, the benefits of employing MMMs for biofilm management are not yet being fully realized or appreciated in the paper and board industry. Regardless of advances in technology, the microbiological insights by MMMs will not be embraced by many until their significance relative to biofilm management are made more transparent. There is a need for an initiative to link biofilm formation, microbiological technologies and disciplinary experts together to reach a common understanding. (Richard et al. 2016, 169-176)

It is widely accepted that biofilms constitute a remarkable portion of the microorganisms in water intensive production processes. The development of biofilms provides numerous advantages to the embedded bacteria, including increases in bacterial resistance to various environmental stresses, sharing of nutrients and metabolic products among bacteria, and facilitation of horizontal gene transfer within the biofilm community. (Richard et al. 2016, 169-176)

Consequently, pathogens in biofilms may potentially survive residual disinfectants and proliferate to higher abundances under oligotrophic conditions in water distribution systems, imposing a threat to public and occupational health. Several other significant problems may also be related to the development of biofilms in these systems, including corrosion of pipes, consumption of residual disinfectants, and generation of tastes and odors. Thus, great concern has been focused on the prevention or removal of biofilms to improve safety in water intensive production processes. (Chao et al. 2015.)

MMMs have provided useful information regarding microbial community structures in biofilms and evaluated the influences of relevant environmental factors and water characteristics, including water temperature, pH, pipe materials, disinfectant concentration, and flow rates. Recently, high-throughput sequencing (HTS) techniques have demonstrated considerable advantages for the analysis of the microbial communities in association with the unprecedented sequencing depth and have been

widely applied to investigations of microbial community structure in various complex environments, biofilms. (Chao et al. 2015)

2 Analyses of bacterial communities

1 Culture-based and DNA-based methods

Cultivation is one of the most fundamental steps in microbiology, and the plate count technique is one of the standard cultivation methods for the enumeration of viable bacteria. Industrial standards are for the assessment of microbial purity and quality and are mainly culture-based (Douterelo et al. 2014, 134-156). However, less than 1 % of the bacteria, for example, in drinking water, is currently culturable and cells of usually culturable bacteria can also exist in a viable but non-culturable state, and are therefore not detectable by culture-based methods if the bacterial cells are present in a viable but non-culturable state (VBNC). VBNC cells are characterized by a loss of culturability on routine agar, which impairs their detection by conventional plate count techniques. This leads to an underestimation of total viable cells in samples. Cultivation methods can lead to underestimation of the amount of bacteria present in the sample. (Luhrig et al. 2015, 99-107). Further more Nikolaki (2013) states that >88% of all bacterial isolates belong to four different phyla *e. g.* Proteobacteria, Firmicutes, Actinobacteria and Bacteroidetes from of whcich most are extremely difficult to cultivate in laboratory conditions.

Culture-independent methods to detect microorganisms have been developed in parallel with traditional culture-based methods ever since the classification of bacteria based on 16S rRNA gene sequences. The development and the prevalence of culture-independent molecular technologies have provided revolutionary progress in microbial studies. The development of these technologies contributes significantly to the research of microorganisms that cannot be detected by traditional methods such as culture-dependent methods. Many MMMs targeting the 16S rRNA gene, such as fluorescence in situ hybridization (FISH) terminal restriction fragment length polymorphism (T-RFLP), denaturing-gradient gel electrophoresis (DGGE), clone library analysis, quantitative PCR, and next-generation DNA sequencing (NGS) technologies, have been applied to various microbial studies. Notably, the advent of NGS

technologies enabled a large-scale research of the bacterial community. It is important to asses the intended method to be used as each method possesses different chrasteristics and principles. Methods differs from specifity, rapidness, cost efficiency and comprehensiveness. Therefore it is important that the methods used in studies are suitable for the objective and materials. (Fukuda et al. 2016, 223-232.)

The differentiation between live and dead bacterial cells presents an important challenge in MMM applications. Due to the persistence of DNA in the environment after cells have lost viability, DNA-based detection methods cannot differentiate whether positive signals originate from live or dead bacterial targets (Nocker et al. 2006, 310-320). Not until the development of DNA binding molecules, such as propidium monoazide, the distinction between viable and dead organisms was not feasible. Methods of detecting active cells are not widely used. Furthermore, the lack of cell activity in a system does not mean that the cells will not become active under more favorable conditions, for example, when more nutrients are available (Oliver 2010, 125-180).

2 Gene sequencing

Amplicon sequencing (Figure 3.) is a highly targeted approach that enables researchers to analyze genetic variation in specific genomic regions. The ultra-deep sequencing of PCR products (amplicons) allows efficient variant identification and characterization. This method uses oligonucleotide probes designed to target and capture regions of interest, followed by next-generation sequencing (NGS). Common application is sequencing the bacterial 16S rRNA gene across multiple species, a widely used method for phylogeny and taxonomy studies, particularly in diverse metagenomics samples. (Wang and Qian 2009.)

The method of Next Generation Sequencing of 16S rRNA gene amplicons provides deeper insight into the community composition than previously mentioned other MMMS. As a single marker, 16S rRNA sequencing ables to capture broad changes in bacterial community diversity, an attractive feature due to the lower cost of sequencing only a small part of the genome. One of the downsides of amplicon sequencing is

that it does not provide information about the potential genomic functions of the community, nor does it detect higher form of life such as molds, yeast or single cell organisms. (Cariou et al. 2018, 461). For this new methods are been developed focusing on ITS reagion or 18s rRNA gene as well as Whole Genome Sequencing (Kukkurainen et al. 2018)

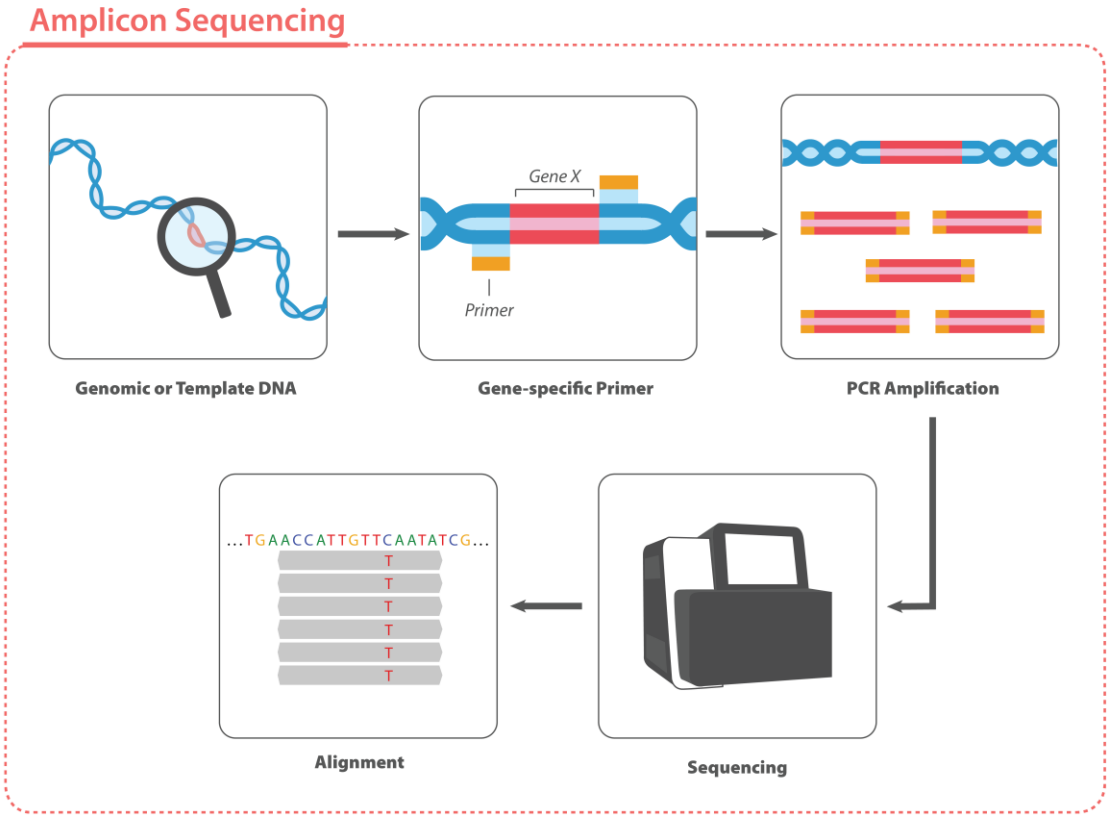

Figure 3. Amplicon sequencing**.** Schematic presentation of amplicon sequencing. First genomic DNA is extracted from the sample. Secondly, the target gene is amplified with specific primers. The produced amplicons of that gene, in this study 16s rRNA gene, is sequenced using Illumina MySeq platform. (ABM, https://www.abmgood.com/Amplicon-Sequencing-Service.html)

The characterization of a single genetic marker, such as the 16s rRNA gene, has been extensively used for assessing the diversity of microbial populations for phylogenetic and taxonomic studies. The 16s rRNA gene exists in all bacteria and is composed of relatively conserved regions. Primers hybridize to the conserved regions for PCR amplification and sequencing of the variable regions, which cluster into operational taxonomic units (OTUs) according to degree of similarity. This classical approach has

yielded comprehensive databases (e.g. RDP) for comparison of sequences in an ecosystem and evolutionary analyses applicable to large projects (Jovel et al. 2016, 459). The classical OTU approach is recommended to be replaced by Amplicon Sequencing Variants (ASVs) due to higher resolution. The OTU is a cluster of sequencing reads that differ by less than a fixed similarity as ASV can be resolved to the level of single nucleotide difference over the sequenced gene region (Callahan et al. 2017, 2639-2643).

In the present work Illumina MiSeq platform is used for the analysis of biofilm formation and bacterial community succession against time in a board machine wet-end splatter area surfaces.

Amplicon sequencing of the 16S rRNA gene, as used in this study, is a method for community analysis, and is not suitable for the identification of bacteria to species level or individual pathogenic bacteria in a complex environment such as board and paper manufacturing process biofilms: "The cumulative results from a limited number of studies to date suggest that 16S rRNA gene sequencing provides genus identification in most cases (>90%) but less so with regard to species (65 to 83%), with from 1 to 14% of the isolates remaining unidentified after testing. Difficulties encountered in obtaining a genus and species identification include the recognition of novel taxa, too few sequences deposited in nucleotide databases…" (Janda & Abbott 2007, 2761-2764).

3 Materials and methods

1 Biofilm by-stream model

Area from the wet end splatter area of a board machine was selected and restricted for the experiment. Metal surface was divided in 6 sectors by 6 columns (1 cm X 10 cm) (See Figure 5.). Initial samples for time point 0 (= mature biofilm) was collected as reference sample. The area was disinfected by rinsing the area with 70 % ethanol followed by 10 % bleach. Residual bleach was removed by purified water wash.

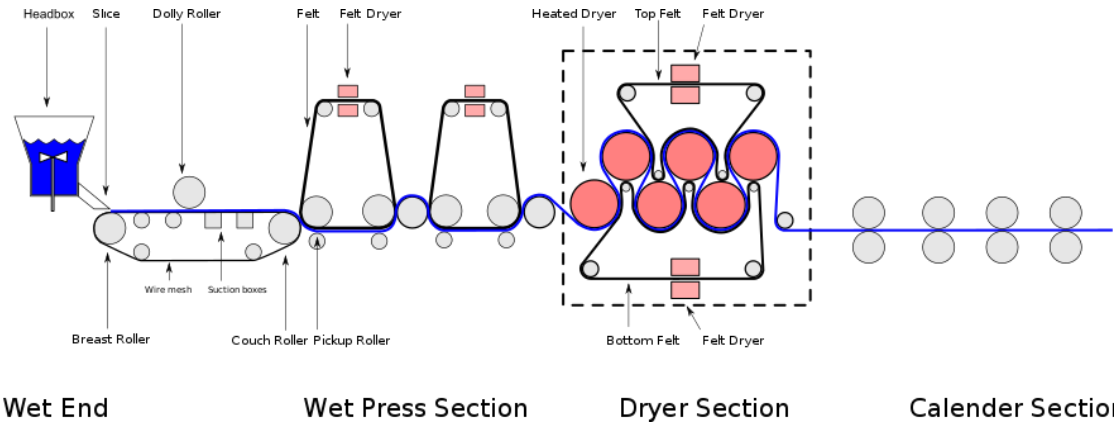

Figure 4. Board machine**.** Diagram showing simplified sections of paper and board machines. (Wikipedia, 2018)

Figure 5. By-stream test site. Splatter area test-site after disinfection. The test site was divided in 6 sectors which were further divided in 6 columns (1 cm X 10 cm). Samples were scraped from the sectors (surface area of 10 cm^2) for each time point in three replicates.

2 Biofilm sampling time points

Sampling of pilot scale by-stream biofilm model at the mill started with 2-week-intervals which turned out too scarce to assess initial attachment and onset of biofilm. In the next experiments samples were collected at weekly intervals but after a thorough analysis the sampling points it were revealed that biofilm was developing as early as 3 days. Daily dynamics were assessed in the following experiments. Even daily dynamics was not fast enough for the initial onset of biofilm and final experiment was conducted on hourly interval sampling. Finally, the spatter area was sampled after 4 months and analysed for long-term dynamics and hence, the validity of the by-stream biofilm model in relation to authentic biofilms. In addition, the machines foils (known to harbour mature biofilm) were collected as a reference to validate the spatter area maturing (Figure 6..).

3 Sample collection

Samples were collected, according to sampling schedule by using a spatula and the scraped biofilm samples were collected into a sample collection tubes containing Tris-EDTA buffer and Proteinase K in pH 8. Samples were transported to Stora Enso Research Centre Imatra and stored in -20 C freezer.

4 DNA extraction

Samples were subjected to Stora Enso internal DNA extraction protocol (Attachment 1; modified protocol that includes phenol-chloroform-isoamylalcohol purification and isopropanol precipitation of DNA followed by ethanol wash steps). DNA was extracted by semi-automated Starlet liquid automation robot (Hamilton Gmbh).

5 PCR and NGS sequencing

The obtained DNA was amplified by using primers designed for the conserved region of 16s rRNA gene (Attachment 3.). The PCR amplicons were further purified by Mobio DNA purification kit and subjected to Illumina DNA indexing protocol (Attachment 2.)

and sequenced by using Illumina V3 sequencing protocol. Sequencing and down-stream bioinformatics was performed by Eurofins MWG and Industrial Water ltd.

6 Quantitative Polymerase Chain Reaction (qPCR)

The DNA samples were analysed by using Stora Enso internal qPCR protocol (Attachment 4;ROCHE SybGreen, ROCHE lightCycler 480, previously developed qPCR primers based on Sanger sequencing of 16s rRNA gene products) to evaluate the quantity of different bacterial clusters as well as the total amount of bacterial 16s rRNA genes in the samples and to confirm the findings from the NGS analysis.

4 Results

NGS data of the mature authentic (n=19) and by-stream model (n=36) biofilms and the mature authentic biofilms from board machine wire change (n=30) (Figure 15.) revealed the diversity of mature biofilms from proteobacteria to bacteroidetes, actinobacteria, deinococci and chloroflexi (Attachment 6).

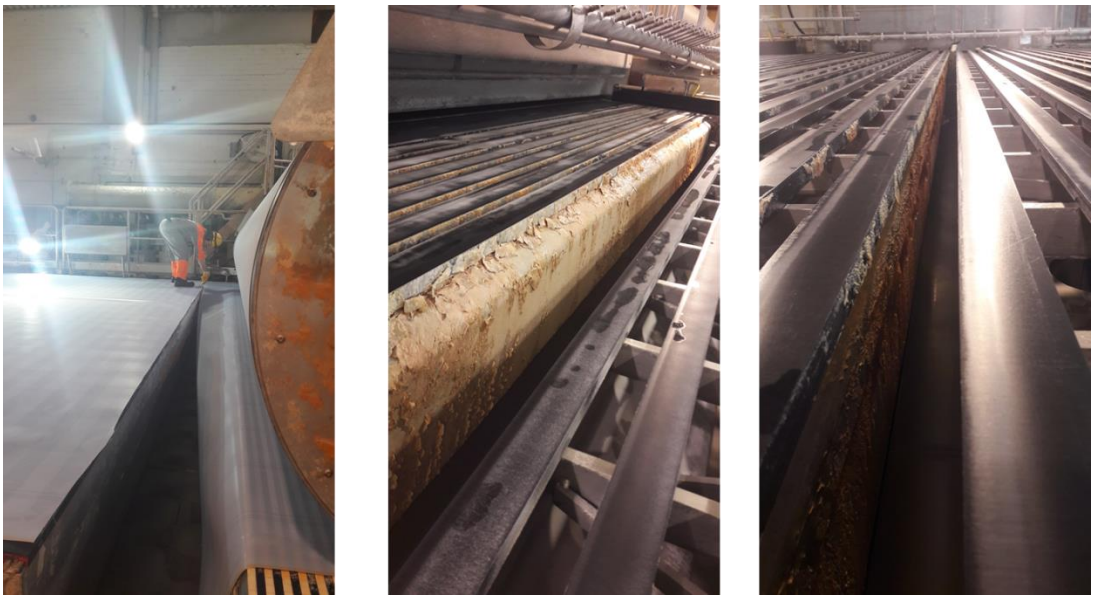

Figure 6. Wire section**.** Wire change during maintenance brake. The biofilm formation is observed in between the foils. The deposits between the foils were sampled and analysed by using NGS and qPCR technique.

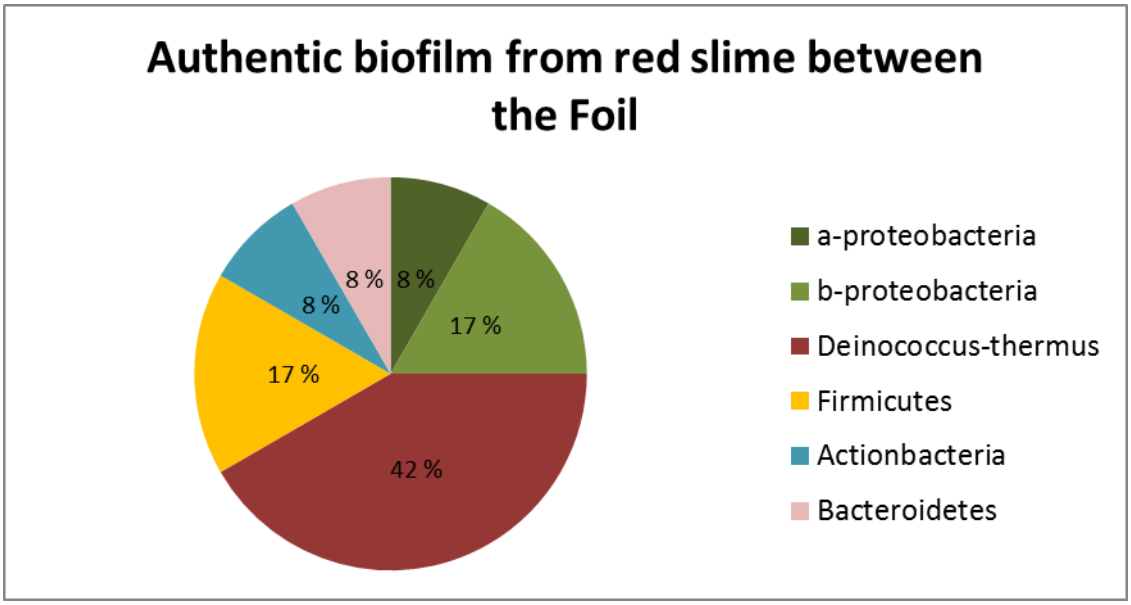

Figure 7. Bacteria in biofilm. 16s Next Generation Sequencing results from the deposits between the foils. Foil biofilm deposits presents mature and older biofilm found from the board machine.

Analysis of all biofilms from daily biofilms to weekly biofilms and further to monthly and even to biofilms which prevailed for years implied that a- and b-proteobacteria colonize the surfaces first and remain the bacterial majority for the first weeks until two or three months. Thereafter, the diversity increases to phyla Bacteroidetes, Actinobacteria, Deinococcus-Thermus and Chloroflexi.

The by-stream system was sampled in hourly intervals. First observation of bacterial attachment to the spatter area by-stream model surface was observed after 6 hours. Analysis of all biofilms from hourly biofilms (Figure 8.) to weekly biofilms (Figure 9.) and further to monthly samples and biofilms which had prevailed for years (in between the foils) implies that a- and b-proteobacteria colonize the surfaces first and remain the majority for the first weeks (Figures 10. & 11.) perhaps until two or three months (Figures 12. & 13.). Thereafter, the diversity increases as representatives of family Chitinophagaceae and Flavobacteriaceae within phylum Bacteroidetes, of order Actinomycetales within phylum Actinobacteria, of family Thermaceae within phylum Deinococcus-Thermus and of families Chloroflexaceae, Aerolineaeceae, Caldilineaceae and Kallotenuaceae within phylum Chloroflexi appear to contribute to the microbial community (Figure 14.).

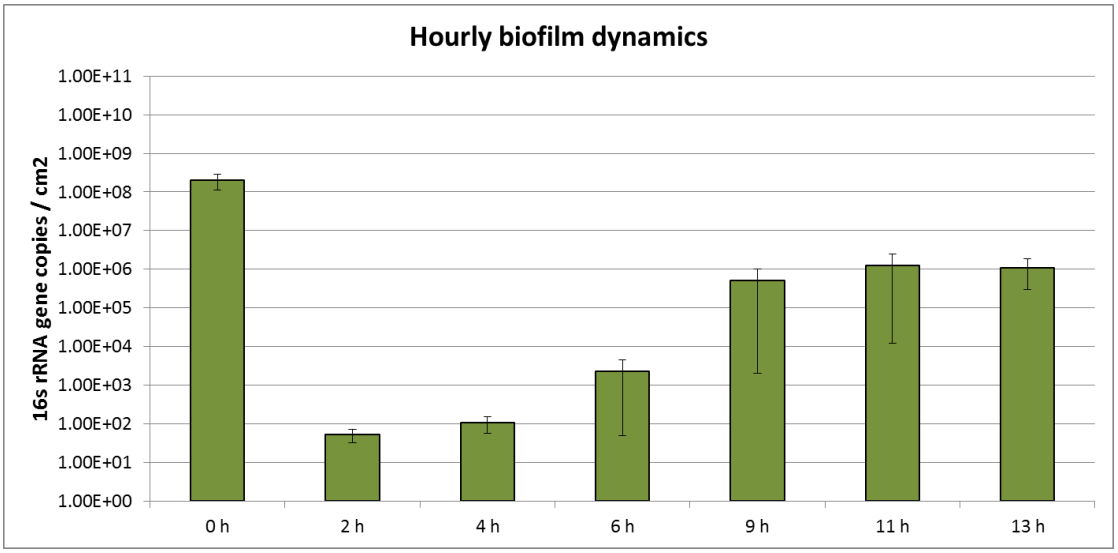

Figure 8. Hourly biofilm dynamics. The by-stream system was sampled in hourly intervals. Results are the mean (n=3) of 16s rRNA gene copies / cm^2. Error bars represents the standard deviation of the mean.

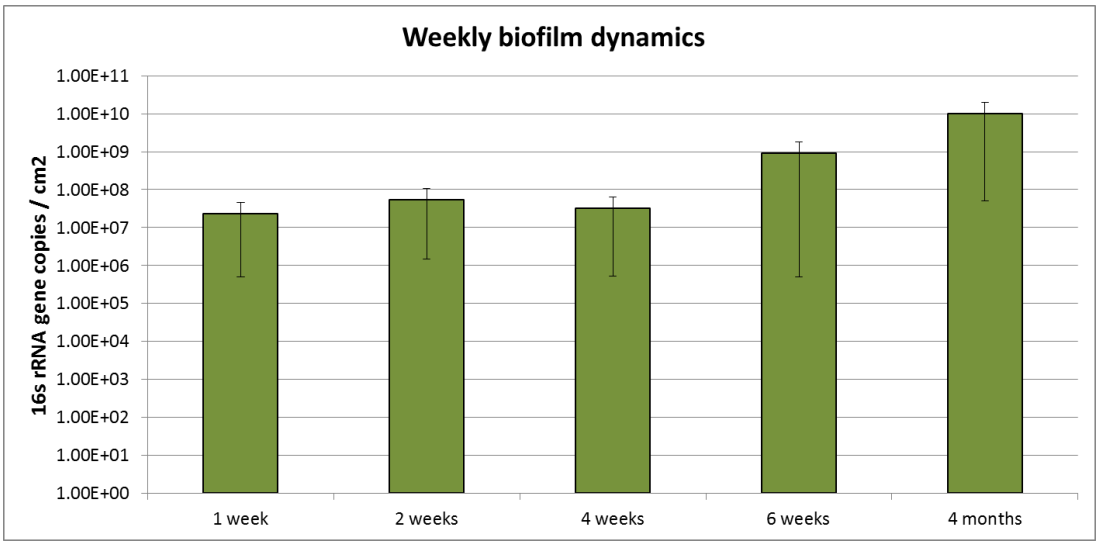

Figure 9. Weekly biofilm dynamics. Weekly biofilm dynamics did not reveille the primary attachment dynamics. Results are the mean (n=3) of 16s rRNA gene copies / cm^2. Error bars represents the standard deviation of the mean.

Pilot scale side-stream biofilm model samples implied that a- and b-proteobacteria are important contributors to biofilm community during the hours of biofilm attachment. Results from daily samplings indicate that a-proteobacteria act as primary colonizers for biofilms attaching to board machine surfaces.

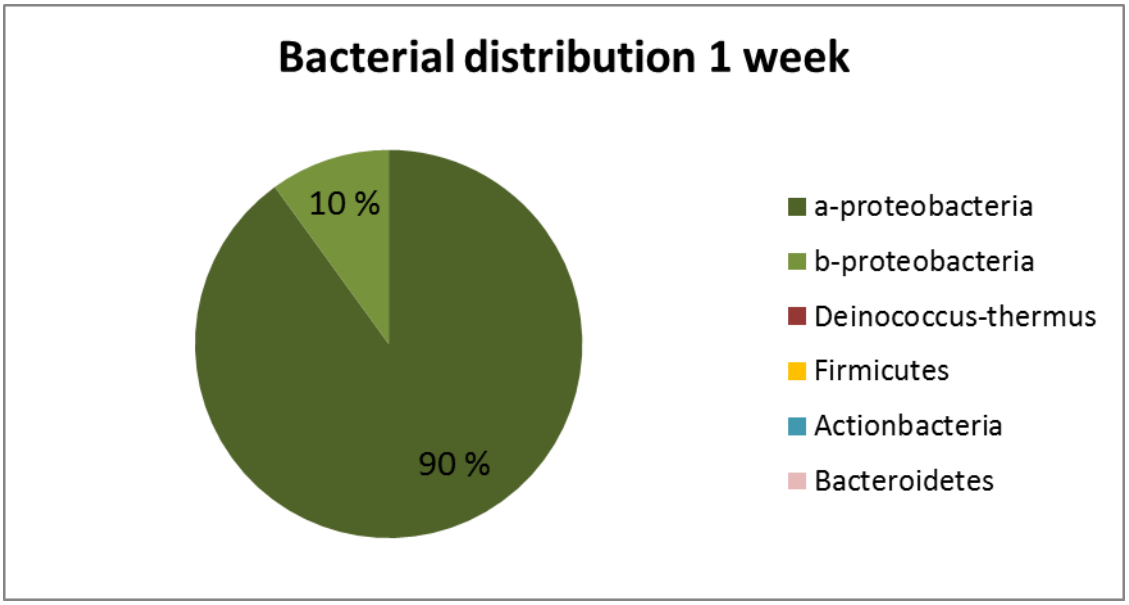

Figure 10. Bacterial distribution 1 week. Biofilm bacterial distribution analysed by qPCR. 6 main bacterial clusters were analysed by pre-designed qPCR primers. In 1 week the major populations present on the surface of the by-stream area were a- and b-proteobacteria.

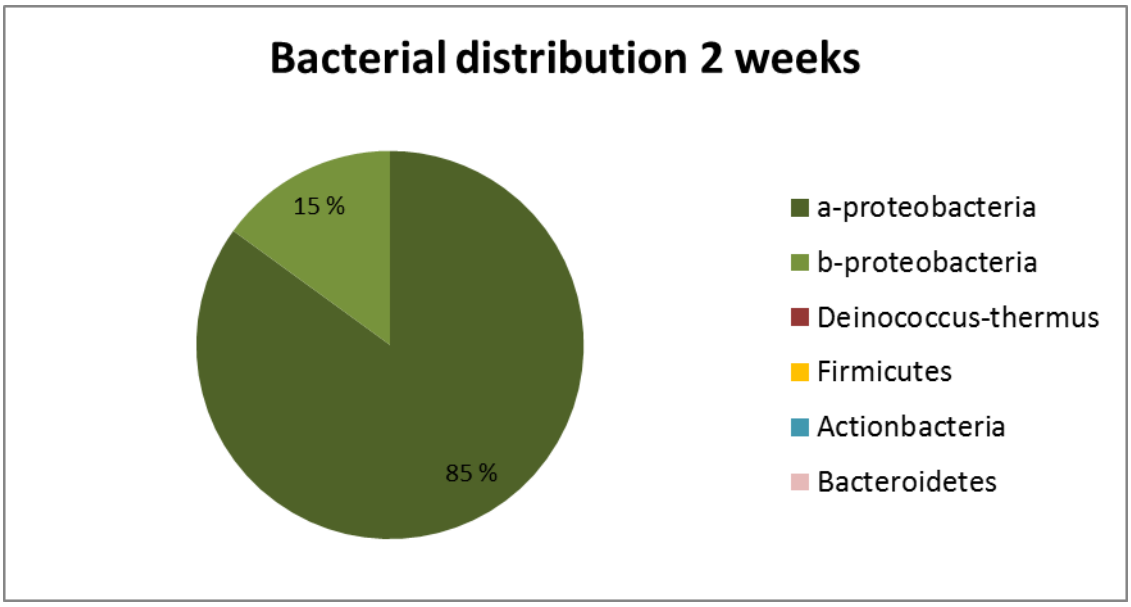

Figure 11. Bacterial distribution 2 weeks. Biofilm bacterial distribution analysed by qPCR. 6 main bacterial clusters were analysed by pre-designed qPCR primers. After 2 weeks the major populations present on the surface of the by-stream area were a- and b-proteobacteria.

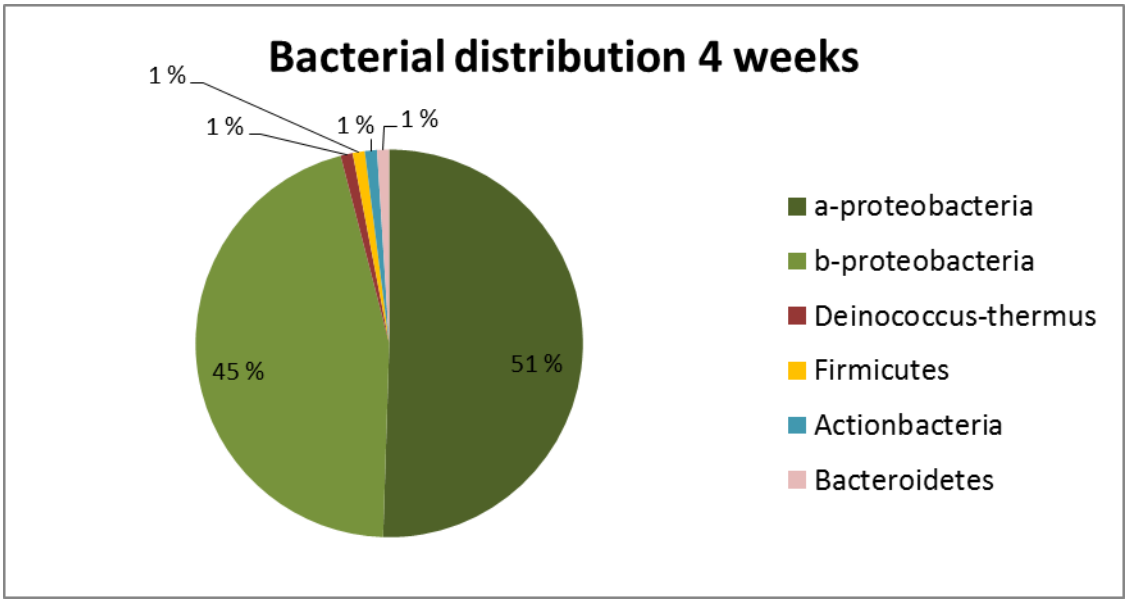

Figure 12. Bacterial distribution 4 weeks. Biofilm bacterial distribution analysed by qPCR. 6 main bacterial clusters were analysed by pre-designed qPCR primers. After 4 weeks the major populations present on the surface of the by-stream area were a- and b-proteobacteria. Deinococcus-thermus, Firmicutes, actinobacteria and bacteroidetes clusters starts to appear on the surface.

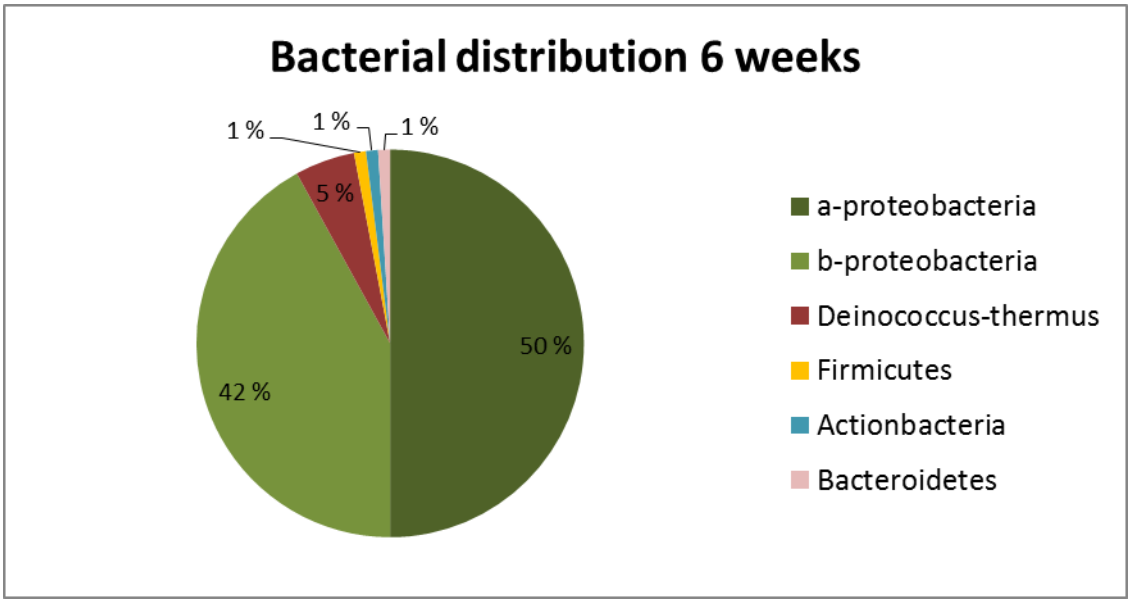

Figure 13. Bacterial distribution 6 weeks. Biofilm bacterial distribution analysed by qPCR. 6 main bacterial clusters were analysed by pre-designed qPCR primers. After 4 weeks the major populations present on the surface of the by-stream area were a- and b-proteobacteria. Deinococcus-thermus, Firmicutes, actinobacteria and bacteroidetes clusters starts to appear on the surface.

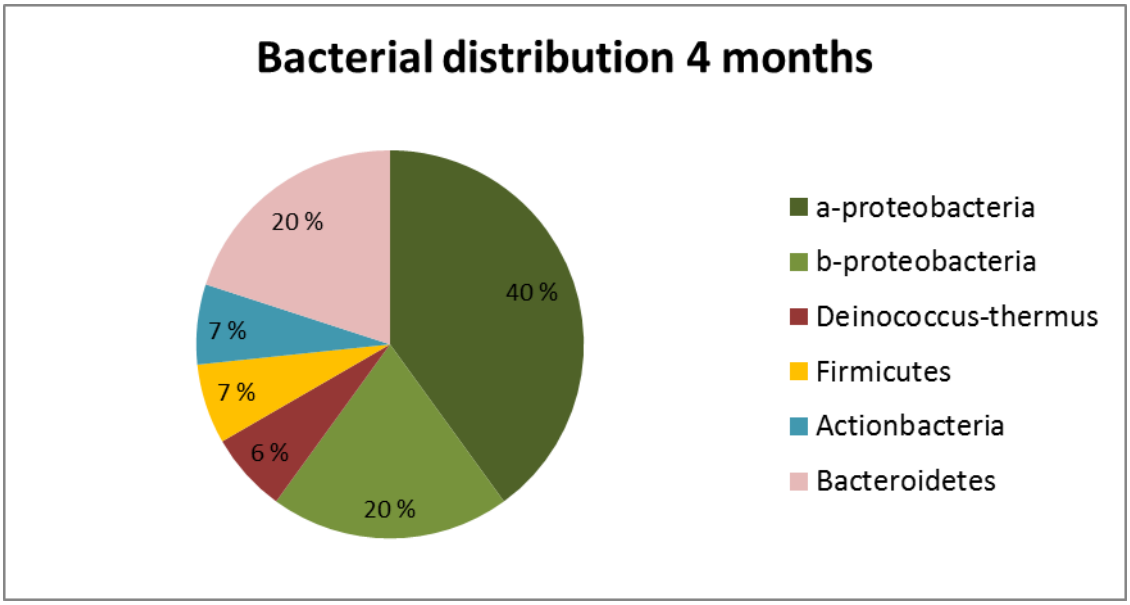

Figure 14. Bacterial distribution 4 months. Biofilm bacterial distribution analysed by qPCR. 6 main bacterial clusters were analysed by pre-designed qPCR primers. After 4 months the bacterial diversity has statistically shifted (Shannon-Wiener index). The most abundant bacterial clusters were a- and b-proteobacteria and bacteroidetes.

Authentic biofilms also emphasized the importance of a-proteobacteria in biofilms, but also other bacteria, including bacilli, deinococci, bacteroidetes, chloroflexi and spirochaeta, were detected as important clusters. Interestingly, same microbial clusters have been later found at two different mill locations (data not shown).

Current hypothesis is that a-proteobacteria is the first cluster to attach the surfaces in paper and packaging board process environment. B-proteobacteria seems to be the next major biofilm cluster, but with time, also other bacteria become dominant in biofilms.

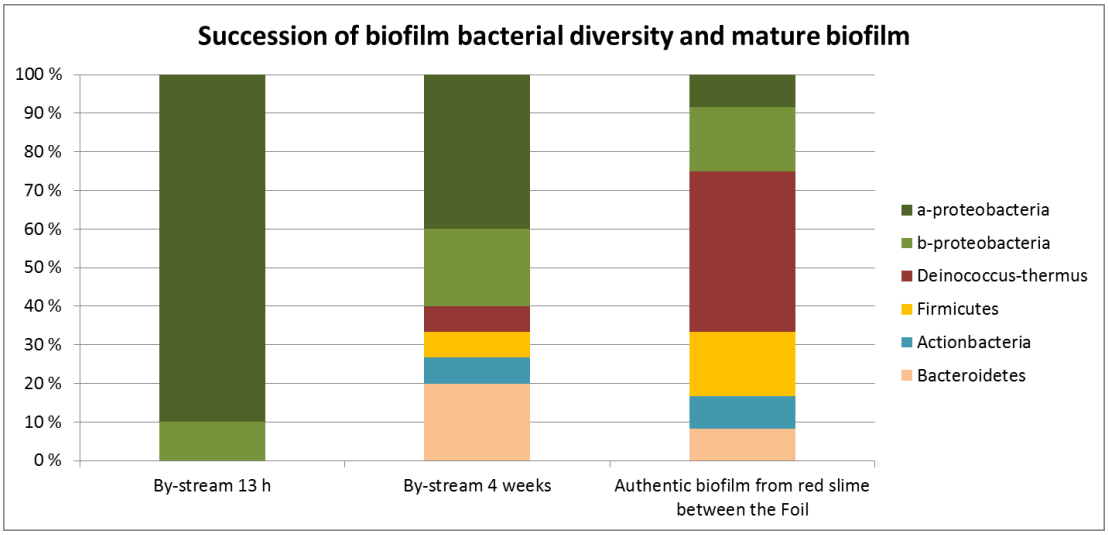

Figure 15. Bacterial succession. NGS bacterial diversity and succession of biofilm bacterial diversity (by-stream area) and mature biofilm from the foil. The diversity of bacteria increases from 4 weeks to mature biofilm.

Otherwise, qPCR analysis was in line with NGS data, but mature biofilms contained bacteria in families Aerolineaeceae, Caldilineaceae and Kallotenuaceae which were not covered by the existing Chloroflexi assay (previously designed based on Sanger sequencing of 16s rRNA gene sequencing). Therefore, the Chloroflexi qPCR assay was updated to cover also these families (Attachment 5).

New qPCR assays revealed that Deinococci are much less abundant in paper making environment than commonly thought (Kolari 2003). Deinococci are traditionally considered responsible for the pink/red slime, but now it appears that these biofilms more often comprise of pink/red proteobacteria and bacteroidetes than deinococci. Nitrogen fixing is initiated 3-4 weeks after biofilm formation (Attachment 5).

Daily scale sampling of the pilot biofilm model indicated a- and b-proteobacteria to be the first to attach to surfaces which in fact is in line with early work by Kolari et al. (2003) even though later research emphasized the role of deinococci in attachment.

Proteobacteria prevail as dominant microbes for ~3 months after which other bacteria start to emerge.

5 Conclusions and discussion

1 Impact of biofilms and new analytical methods

The project aimed at finding solutions for the prevailing biofilm issues at the mills. Not only the key dynamics of biofilm attachment and maturation were revealed, but information from the process conditions were obtained. The information was used to solve numerous root causes of biofilm formation challenges. The presented work relates to any processing environment that are water intensive and uses natural raw materials as a part of manufacturing. The need for renewable and ecological material is increasing globally. Solution portfolios for sustainable packaging material to replace plastic consumables are growing. The upscaling of production by less resources leads to more efficient use of biological raw materials. Biological material is prone to accumulate adverse effects regarding human health, production efficiency and product safety and purity. On the other side, consumers are more aware of the quality requirements of the products that are replacing products from fossil origin. Therefore it is important to identify and manage the adversities that production of renewable materials poses. The current bio economic and circular economy trends tend to increase the need of new analysis technologies and management strategies to avoid these adverse effects explained in this work.

During the experiment critical points for the means of investigation were identified. It cannot be emphasized that the ambitious plan was to fall in multiple pits of un-identified problems thus consuming energy and resources to climb of from these falls. The results and methodology also needs a critical view. There are the issue of sample handling and sample integrity, DNA extraction efficiency and as described previously the bias of primer selection when broad range primers are used in the amplicon PCR step of the NGS method. Furthermore, the validity of reference libraries where the sequencing data is compared can be critically reviewed. When we retrospectively re aligned the sequences and did library alignment the results deviated from the original bioinformatics pipeline results. Reference databases collect vast amount of changing information and the phylogeny of the bacterial species might be re allocated to, for

example different genera, therefore it is recommended to revisit the analysis results periodically and re-analyse the bioinformatics pipeline for upcoming research projects.

2 Prevention of biofilm formation

Bajpai (2015) summarizes the preventio of biofilm formation as “Good housekeeping and regular inspection of all areas, effective boilouts, and regularly scheduled washups reduce slime development. Conventional slime control methods generally employ combinations of biocides. Alternative control measures use enzymes, biodispersants, bacteriophages, competing organisms, and biological complex formers. Using enzymes for slime control is expected to bring important benefits to the pulp and paper industry. Enzymes represent a clean and sustainable technology: they are nontoxic, readily biodegradable, and are produced using renewable raw materials. Use of enzymes in combination with biodispersants appears to be a promising method for slime control.” Also new strategy to deprive one key nutritional element from the water circulation during manufacturing process was discussed.

These strategies and experiments will continue after the finalization of the BioM project. Research Centre Imatra will continue to provide the Microbial Management Program (Attachment 5.) service model for internal and external clients.

Very few studies using NGS have been performed on eukaryotes in biofilms such as fungi and amoeba or protozoa (Douterelo et al. 2016, 3301-3311) although they are an important part of the biofilm community (Buse et al. 2013, 219-225). Questions regarding fungi (unicellular yeasts and multicellular moulds) are increasingly presented in discussions and meetings with Stora Enso customers. As a food packaging material supplier, Stora Enso needs the capability and know-how to answer these questions and manage bacteria and fungi in the process to guarantee end product hygiene. In-house expertise in analytical methods and interpretation of results as well as safety and legislative issues are crucial to support production processes and guarantee end product hygiene level.

So far, fungal communities have not been reliably assessed due to the lack of appropriate research tools. Prokaryotic research has progressed substantially in recent

years, but eukaryotic microorganisms are a new area of research. The effects of fungi in the manufacturing process remain currently unknown.

The research focused on the understanding of the contamination sources and process control for the prevention of biofilms. The need for fast solutions to optimize process conditions and make quick executive decisions in the paper and packaging making process to avoid microbiological growth and risks was to be natural course for the project to move towards.

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6 Attachments

1 Stora Enso in-house DNA extraction protocol

2 List of sequencing primers

3 List of qPCR primers

4 qPCR protocol

5 Stora Enso Microbial Management Program database 2017

6 Table of results